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MORPHOLOGICAL AND BIOCHEMICAL VARIATIONS OF *FUSARIUM OXYSPORUM* INFECTING FCV TOBACCO IN KARNATAKA

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ABSTRACT

Fusarium wilt is one of the devastating diseases of tobacco caused by *Fusarium oxysporum* which is a soil borne plant pathogen. Fourteen isolates were isolated from Hunsur, Piriyapatna belt 1 & 2 and H. D. Kote. These isolates exhibited morphological variations on PSA media. Further these isolates were subjected to SDS PAGE and variations were found prominent. The molecular weight of all protein bands ranged from 14.3 kDa to 97.4 kDa. Isolate 6FON was found to be distinct in having the band of 70.79 kDa. 6FON and 12FON were found unique in the band formation and with the molecular weight of 66.07 kDa. 12FON and 13FON isolates showed similar banding patterns and molecular weight ranged from 26.92 kDa to 82.28 kDa. These 14 isolates were grouped into five *viz.*, 10FON, 13FON, 9FON, 5FON & 1FON. These 5 isolates were tested for Carling compatibility reaction. They also showed variation in their compatibility. Presence or absence of barrage line between the isolates grown in a Petri plate was taken as criteria to assess the compatibility. Among the five representative isolates, only 13FON and 5FON combinations showed compatibility.

KEYWORDS: *Fusarium oxysporum*, SDS PAGE, Variability, Carling Reaction

INTRODUCTION

Fusarium species have been important for many years as plant pathogens causing diseases such as crown rot, head blight, and scab on cereal grains, vascular wilts on a wide range of horticultural crops, root rots, cankers, and other diseases such as pokkah-boeng on sugarcane and bakanae disease of rice (Gilardi *et al.,* 2007; Kausar *et al.,* 2009; Nelson, 1981; Tawfik and Allam, 2004). These are a widespread cosmopolitan group of fungi and commonly colonize aerial and subterranean plant parts, either as primary or secondary invaders. Some species are common in soil and they result in necrosis of roots of many agricultural crops (Nelson, 1983). Of all diseases caused by *Fusarium*, probably the most important ones are the vascular wilt diseases caused by the *formae specialis* of *Fusarium oxysporum*. Fusarium wilt is one of the devastating diseases of tobacco and has become a threat to its cultivation.

F. oxysporum is known to exhibit cultural and morphological variations (Chittem K, Kulkarni, 2008; Ho and Varghese, 1988; Lucas, 1975). However, subculturing of the genus consecutively may lead to many more variants (Bai, 1996). In most of the species small patches or sectors were formed and they differed from their parental colony (Ho and Varghese, 1988). Different isolates may show cultural and morphological differences when grown on the same media (Leslie and Summerell, 2006). This type of work will help to know the extent of variations a pathogen exhibits and also the variations existing among the isolates which are collected from a particular geographical area (Dudley *et al.,* 2005; Shenoi *et al.,* 2004).

Vegetative compatibility in fungi relates to its morphological differences and inter-relationships among the individuals and population respectively. Here the strains of a species which have different loci can fuse to form a

heterokaryon. Those which form heterokaryons and exhibit the stability, this can be considered as, the strains that belong to the same vegetative compatibility group. On the contrary, if the strains have different loci, they belong to different group. Tests in Petri dishes make it relatively easy to determine the VCG's for a population of isolates and this provides a robust view of population diversity (Burgess *et al.*, 2001; van Heerden *et al.*, 2001). Where it is possible to define the VCG's of individuals in the population of fungi, such tests are relatively simple and inexpensive. This approach also provides a means of understanding the genetic diversity in a fungal population where molecular markers and the concomitant sophisticated facilities may not be readily available.

Although the taxonomy of plant pathogenic fungi mainly depends on morphological and pathological criteria (Kistler, 1997), biochemical markers are also useful when it is correlated with the morphological characteristics. Mycelial differences in protein pattern among *Fusarium* spp. and their isolates have been reported by various workers (Balali GR, Iranpoor, 2006; Kumar *et al.*, 2010; Sharma *et al.*, 2005). Electrophoretic analysis of proteins can be used as an adjunct to morphological, cultural and vegetative compatibility groups of different isolates of the pathogen (Hall, 1967). Protein on PAGE provides a well established and efficient tool for revealing genetic variability in fungal population (El-Kazzaz *et al.*, 2008). The assessment of genetic diversity of *F. oxysporum* is required for the development of long-term disease management strategies. Studies on genetic relationship and phylogeny among *Fusarium* species were conducted at the protein and DNA levels (Huss *et al.*, 1996; Matsuyama and Wakimoto, 1997). The information regarding variability in protein content of the wilt pathogen is lacking to KLS region of Karnataka, India. In the present study, an attempt was made to find the diversity of *Fusarium oxysporum* f. sp. *nicotianae* (FON) using total soluble protein profile.

MATERIALS AND METHODS

Cultural Studies

The isolates that were isolated from infected tobacco plant was brought down to pure culture which were 14 in number. They were cultured on Potato Sucrose Agar (PSA) medium. This medium was prepared by adding sucrose (20gm), Agar (20gm) to the potato extract (200gm in 500ml), pH was maintained at 6. Twenty mililiter of the medium was poured to 90mm Petri plates. Such Petri plates were inoculated with 5mm Diam disc cut from periphery of actively growing culture and incubated at 26±2°C for 7 days. All 14 isolates were preserved by soil preservation technique and were subcultured for further compatibility and protein studies.

FUNGAL GROWTH AND PROTEIN EXTRACTION

Fourteen isolates were grown on Potato sucrose agar (PSA) in 250 ml conical flask for 20 days and the mycelial mats were collected on Whatman No. 1 filter papers and washed with sterile deionized distilled water to remove extraneous contaminations. Such mats were blot ried. 0.1gm of mycelial mats of each isolate was macerated with 1ml of extraction buffer (100 mM TrisHCl, 1mM EDTA, 10mM KCl, 10mM $MgCl_2$ supplemented with 4% PVP). The homogenenate was centrifuged at 20,000Xg for 10min using Remi laboratory centrifuge (Vasai, India). The clear supernatant was collected into fresh microfuge tubes and stored at -20°C until use.

TOTAL SOLUBLE PROTEIN DETERMINATION

Protein determinations of all isolates were done by Lowry's method (Lowry *et al.*, 1951).

Sample Preparation

Fresh mycelial extracts were used every time. To 50µl of sample (containing approximately 40µg of protein), 50µl of sample buffer was added and heated at 96°C for 3minutes. Denatured proteins were cooled at room temperature and

quick spinning was performed before electrophoresis. 50µl protein sample was loaded in each well. Standard protein marker was loaded in one of the wells.

Electrophoretic Conditions

Electrophoresis was carried out at room temperature (26±2°C) using Tri-Glycine buffer (tank buffer) at pH 8.3. Gels were run at 20mA constant current for approximately 4-5h. Until the tracking dye bromophenol blue reached the end of the gel.

Staining and Destaining of Gel

The gels were stained in a mixture of Coomassie Brilliant blue R250 for 4-5h and destained repeatedly in the destaining solution. The gels were stored in gel storing solution.

Estimation of Molecular Weight

Protein used as molecular marker standards were Phosphorylase b, 97.4kDa ; Bovine serum albumin, 66kDa; Ovalbumin, 43kDa; Carbonic anhydrase, 29kDa; Trypsin, 20.1kDa; lysozyme, 14.3kDa were obtained from Messrs Sigma chemical Co., USA.

The Rf values of all the proteins were calculated. The molecular weight of standard protein was plotted against the Rf of the standard protein migrated. The molecular weight of protein bands was determined (approximate values) with reference to this curve.

Compatibility Studies

Preliminary investigation on compatibility testes were carried out. Five representative isolates *viz.*, 10FON, 13FON, 9FON, 5FON & 1FON were selected for the study based on their morphological variation. These representative isolates were inoculated to the Potato Dextrose Agar (PDA) medium at 4cm distance. The plates were incubated as above and observed at regular intervals.

RESULTS

Cultural Studies

All isolates showed typical characteristic features of *Fusarium* culture exhibiting varied pigmentation and sector formation on PSA medium. Morphological characteristics of 14 isolates on single medium showed variations in mycelial growth, colony diameter, pigmentation and sector formation and sporulation. Pigmentation ranged from magenta pink to dark pink in upper side and pale violet to dark violet on reverse side of the medium (Figure 1).

Figure 1: Fourteen Isolates of *Fusarium oxysporum* on Potato Sucrose Agar (7-day-old)

FUNGAL GROWTH AND PROTEIN EXTRACTION

Total Soluble Protein Determination

Soluble mycelial protein was estimated by Lowry *et al.*, method from seven-day old culture. Protein content was found to be varying in all the 14 isolates. Isolates 1FON, 2FON, 3FON, 4FON, 5FON, 6FON, 7FON, 8FON, 9FON, 10FON, 11FON, 12FON, 13FON and 14FON showed 15µg, 17 µg, 8 µg, 10 µg, 23 µg, 20 µg, 52µg, 28µg, 39µg 14 µg , 62, 68µg and 58 µg of protein respectively (Figure 4)

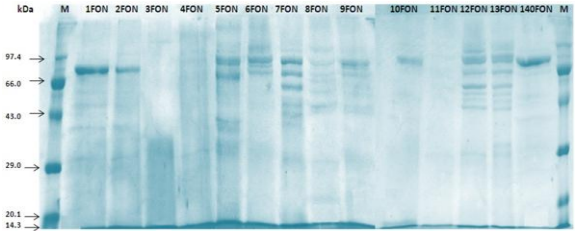


Figure 4: SDS-PAGE Protein Profile Showing Variability in 14 Isolates of *Fusarium oxysporum* f. sp. *Nicotianae*

SDS PAGE

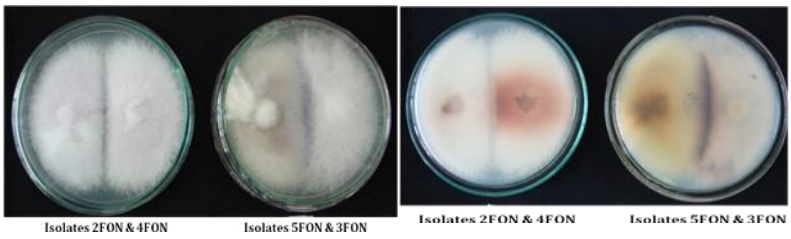
The results showed that each of *F. oxysporum strains* had their own unique protein profiles. The molecular weight of all protein bands ranged from 14.3 to 97. 4 kDa. Isolate 6FON was found to be distinct in having the band of 70.79kDa. 6FON and 12 FON were found unique in bands of 66.07kDa.12FON and 13FON isolates showed similar banding patterns and molecular weight ranged from 26.92 to 82.28kDa. Similarly isolates 1FON and 2FON exhibited similar bands that ranged from 27.54kDa to 69.18kDa in their molecular weights. Isolate 5FON exhibited the bands of 40.74kDa and 36.90kDa along with 4FON and 3FON respectively. Isolates 4ON, 7FON, 8FON 9FON, 10FON, 11FON, 12FON and 13FON exhibited a common band of 43.0kDa. Isolates 3FON, 5FON, 6FON, 9FON, 10FON, 11FON and 13FON showed a common band of molecular weight 30.92kDa which were absent in others (Table 1).

Table 1: Rm Values of Protein Bands of Mycelial Extract of the 14 Isolates of *Fusarium oxysporum* f. sp. *Nicotianae*

Bands	Standard Markers	1FON	2FON	3FON	4FON	5FON	6FON	7FON	8FON	9FON	10FON	11FON	12FON	13FON	14FON	Mol. wt. in kDa
1	1.6															**97.4**
2								0.16	0.16	0.16		0.16	0.16	0.16		81.28
3					0.2	0.2					0.2	0.2	0.2	0.2		75.86
4								0.21	0.21	0.21						74.13
5							0.23									70.79
6		0.25	0.25		0.25		0.25					0.25	0.25	0.25	0.25	69.18
7	0.28						0.28						0.28			**66.0**
8					0.3			0.3	0.3	0.3				0.3		63.10
9					0.33		0.33			0.33	0.33				0.33	58.88
10	0.36				0.36			0.36	0.36	0.36	0.36	0.36	0.36	0.36		**43.0**
11													0.4	0.4	0.4	42.97
12		0.41	0.41			0.41		0.41	0.41	0.41		0.41				42.84
13													0.43	0.43		42.92
14													0.46	0.46		42.77
15		0.5	0.5		0.5	0.5		0.5	0.5						0.5	42.66
16					0.53	0.53										40.74
17				0.58												37.15
18						0.61	0.61									36.90
19		0.63	0.63	0.63	0.63											33.88
20													0.65	0.65		32.36
21				0.66		0.66	0.66			0.66	0.66	0.66		0.66		30.92
22	0.7				0.7	0.7	0.7	0.7	0.7	0.7						**29.0**
23		0.73	0.73													27.54
24								0.75		0.75	0.75	0.75	0.75			21.88
25	0.91															**20.1**
26	0.96															**14.3**

COMPATIBILITY STUDIES

A barrage line was observed in the Petri plates inoculated with isolates 2FON & 4FON and 5FON & 3FON (Figure 2 a&b). The vegetative compatible isolates *i.e.*, isolates 1FON & 2FON and 12FON &13FON, did not show the barrage line and the mycelia of both the isolates were found merged in obverse and reverse sides of Petri dishes (Figure 3 a&b; Table 2).



a. Top View of the Petri Plate **b. Bottom View of Petri Plate**

Figure 2: Isolates 2 FON & 4 FON and 5 FON & 3 FON Showing the Barrage Lines at the Centre

a. Top View of the Petri Plate **b. Bottom View of Petri Plate**

Figure 3: Isolates 1FON & 2FON and 12FON & 13 FON Showing Compatibility (No Barrage Line)

Table 2: Carling Reaction of Five Representative *Fusarium oxysporum* Isolates

Isolates	10FON	13FON	9FON	5FON	1FON
10FON	+	-	-	-	-
13FON	-	+	-	+	-
9FON	-	-	+	-	-
5FON	-	+	-	+	-
1FON	-	-	-	-	+

DISCUSSIONS

The present investigation revealed significant variations in the cultural characteristics, inter-compatibility and protein profile of 14 isolates collected from different geographical area that helped to study the genetic diversity of field populations. This led a base for knowing the distribution of the isolates and its diversity in special distribution. Variations observed included difference in the mycelial growth, colony diameter, pigmentation, sector formation and extent of sporulation. Similar observations were made on PSA for the genus infecting tomato (Booth, 1977; Rini and Sulochana, 2007). During the present study these features helped in designating the Fusarium wilt fungus to *F. oxysporum* (Booth, 1977; Rodrigues and Menezes, 2005).

Colony diameter varied among all the isolates during the study. The results were similar to who isolated the fungus (*F. polyphialidicum)* from the plant debris of South African soil, and who worked on *F. mangiferae* infecting mango. In terms of pigmentation the colonies exhibited an initial light pink colour on PSA, which later turned to various colours on reverse side of petriplates (Marasas *et al.*, 1986; Iqbal *et al.*, 2008). Similar pigmentation in different *forma specialis* of *F. oxysporum* was also observed (Siddique *et al.*, 2010; Chittem and Kulkarni, 2008).

Sector formations were seen to a greater extent on PSA. This result was similar to those who observed the same phenomenon in *F. oxysporum* isolated from oil palm. Sporulationwere also seen varying among the isolates (Ho and Varghese, 1988; Leslie and Summerell, 2006).

Variation in the protein concentration among the isolates was observed in the present study. Similar observations on variations in the protein content were made on *F. oxysporum* f. sp. *carthami* (Raghuwanshi and Dake, 2005). In the present study, the SDS-PAGE also showed variation among the isolates and this may be a supporting data for the presence of high genetic diversity. Similar studies were also found reported (Ibrahim, 2003). This is the first report on the diversity of FON based on SDSPAGE. Such variations based on SDS-PAGE in the case of another *formae specialis viz.*, *F. oxysporum cubense* is also reported (Kumar *et al.,* 2010). Moreover, in some cases the results obtained by SDS-PAGE of whole-cell proteins can discriminate at the same level as DNA fingerprinting (Brown, 1996; Priest and Austin, 1993). The present study showed that fourteen isolates of FON collected from different locations of Karnataka were composed of heterogenous group. Similar type of work was carried out by in *Phytophthora cryptogea* (Bielenin *et al.,* 1988). During the present investigation, biochemical characteristics of the FON isolates colonizing tobacco were varying to a greater extent. This study provides some preliminary information about the population structure and variability among the isolates of *F. oxysporum.*

Although our sample size was insufficient to make any inferences concerning the contribution of the observed genetic diversity to population structure in this study, several relationships between protein profiling and somatic compatibility data were used as to tool to assess the extent of variability. The genetic relatedness was studied by observing the pairing of the two mycelia which accounted for the intensity of hyphal interactions. The isolates in the present study showed that they are vegetatively compatible and incompatible as they have same and different loci respectively (Burgess *et al.,* 2001; Ceresini *et al.,* 2002).

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